RONALD A. DIERWECHTER*

Department of Anatomy, Yale University School of Medicine

INHIBITION OF MAMMARY GROWTH BY HIGH DOSES OF ESTROGEN**

The administration of large doses of estrogen to young male mice was followed by less extensive mammary gland growth than when small amounts of estrogens were given.[1,2] The areas occupied by the mammary glands of the mice given large doses were often but little larger than the glands of untreated controls, but unlike the controls their mammary glands, although very small, had a few alveoli on the short, distended mammary ducts. Mammary glands of restricted size were also observed in monkeys,[2,3] rabbits,[4] dogs,[5] and rats[6] given large doses of estrogens. The area of proliferation of mammary glands of male mice was inversely proportional to the amount of estrogens given beyond a certain optimal amount.[7]

The restricting effect of large doses of estrogen upon the area of non-neoplastic mammary growth might be compared to the effects of large doses of estrogen on neoplastic growth, especially in post-menopausal women.[8] In neither instance has the mechanism for the inhibition or restriction of growth been explained adequately.

Whether the large amounts of estrogen inhibit the growth of mammary parenchyma by direct action or action upon the stroma, or whether the effect is upon the pituitary gland or adrenal glands and hence upon the mammary glands indirectly, is not known. Some experiments to determine how large doses of estrogens inhibited mammary growth were undertaken.

MATERIALS AND METHODS

Male mice of the inbred CBA and C_3H strains, first generation hybrid mice (CC-C_{57} x CBA), and second generation hybrid mice (CC x CC) were used in these experiments. Because the mammary gland and other responses to the hormones that were used at different dose levels were comparable among all genotype groups of mice, genetic and age influences

* Present address: R.F.D. #3, Storm Lake, Iowa.

** Part of thesis submitted as a requirement for the Degree of Doctor of Medicine. This work was in part undertaken under a Josiah Macy, Jr. summer fellowship and supported in part by grants from the United States Public Health Service (C343), the Jane Coffin Childs Memorial Fund and the Anna Fuller Fund administered by W. U. Gardner.

Received for publication 1 November 1961.

within the ranges used were of no significance and will not be given further consideration in the report of the observations. The mice ranged from 15.1 to 23 gm. in weight at the start of the experiment and from 39-54 days in age. Most of the mice were treated for periods of three weeks.

The hormones* used were:

1. Estradiol benzoate (eb) dissolved in sesame oil so that 2 μg. was contained in 0.05 ml. Two μg. were injected subcutaneously every other day.
2. Estradiol benzoate in pellet form (EB). Each compressed pellet weighed 2-3 mg. and was implanted subcutaneously through a trocar.
3. Growth hormone (GH) was obtained in the desiccated form and dissolved in saline just before use so that 0.05 ml. contained 0.1 mg. This was injected daily.
4. Lactogen (L) was also obtained in desiccated form, and dissolved in saline so that 0.05 ml. contained 1 mg. This dose was injected subcutaneously once daily.
5. Progesterone (P) was compressed into pellets weighing about 10 mg. each. Two pellets were implanted subcutaneously through a trocar at the beginning of appropriate experiments.
6. Cortisone acetate (CA) supplied as a suspension was diluted so that 0.10 ml. of suspension would contain 125 μg. of hormone. This amount was injected subcutaneously daily.
7. Adrenocorticotrophic hormone (AC) was injected subcutaneously at doses of either one or two units daily (0.05 ml. of suspension contained one unit of ACTH).
8. Relaxin (CR) was dissolved in benzopurpurine, so that 0.10 ml. of solution contained 100 guinea pig units. This amount was injected subcutaneously every four days.
9. Protamine Zinc Insulin, U.S.P. (I) was diluted with saline so that 0.05 ml. contained 0.5 units. This amount was injected daily.
10. Desoxycorticosterone acetate (DO) was injected in daily doses of 250 μg./0.05 ml.
11. Hyaluronidase (H) was dissolved in saline so that 0.10 ml. contained 1.5, 3.0 or 10.0 viscosity reducing units, and injected daily subcutaneously.

A method for determining the areas of the mammary glands was devised to quantitate and compare the mammary responses. After the skin and subcutaneous tela containing the mammary glands were removed, the skins were stretched uniformly over cork boards and fixed in Bouin's solution. After fixation, the glands and subcutaneous tela were removed from the

* The hormones and chemicals used in this investigation were: Estradiol benzoate, No. 14855, Organon, Inc., West Orange, New Jersey. Growth Hormone, Boving (SOMAR-A) Lot R50109, Armour Laboratories, Kanakee, Illinois. Lactogenic Hormone (PANLITAR), The Armour Laboratories, Lot No. R10109. Progesterone, USP, Lot C2098, Mann Research Laboratories, Inc., New York, N. Y. Cortisone acetate (CORTONE), Merck & Company, Inc. ACTH (CORTROPHIN-ZINC), Organon, Inc., West Orange, New Jersey. Relaxin, Releasin R, Warner-Lambert Research Lab., Morris Plains, New Jersey. Protamine Zinc Insulin, Eli Lilly & Company, Indianapolis, Indiana. DOCA (CORTATE), Schering Corporation, Bloomfield, New Jersey.

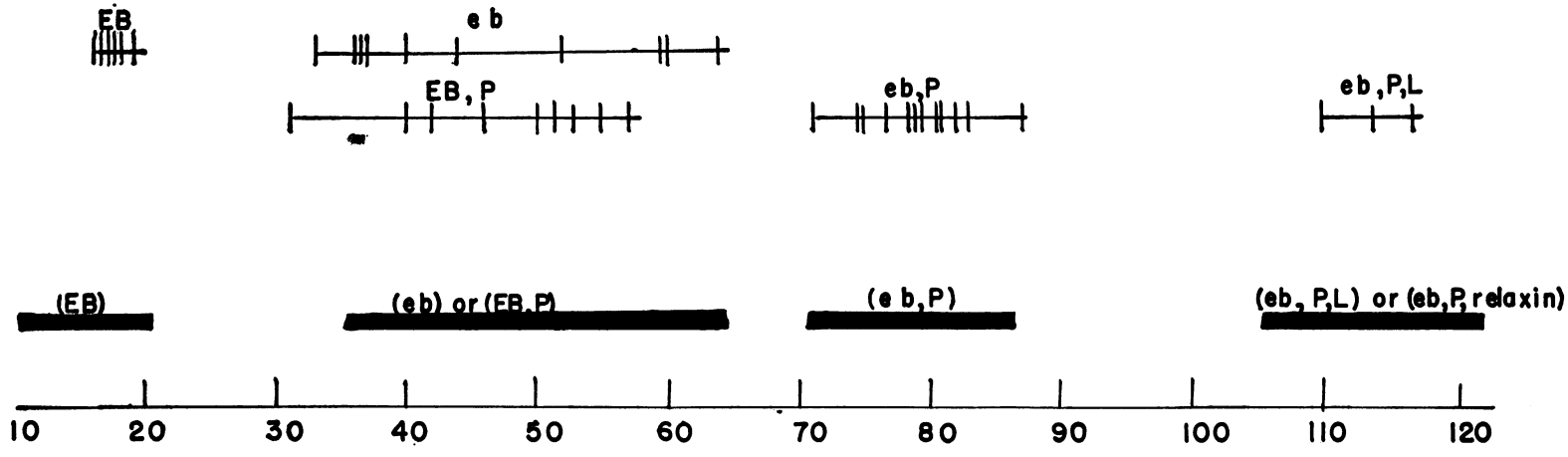


FIG. 1. Relative sizes in terms of weight units of mammary glands of mice given different combinations of hormone treatment (EB, large doses of estradiol benzoate; eb, small doses of estradiol benzoate; P, progesterone; L, lactogen). The wide lower horizontal bars represent the range of "weight units" for each basic type of treatment. The upper horizontal bars and the shorter cross lines present the distribution of weight units within the range of the mammary weight units for individual experiments.

skin, stained in Mayer's hemalum, suitably de-stained, dissected from extraneous tissue, and prepared for mounting on glass slides after dehydration and clearing in xylol.

Each slide so prepared was placed individually in a photographic enlarger, as one would place a negative film for printing, and prints were made on photographic paper at a uniform enlargement. The mammary gland images were then cut out to include the area of paper within the

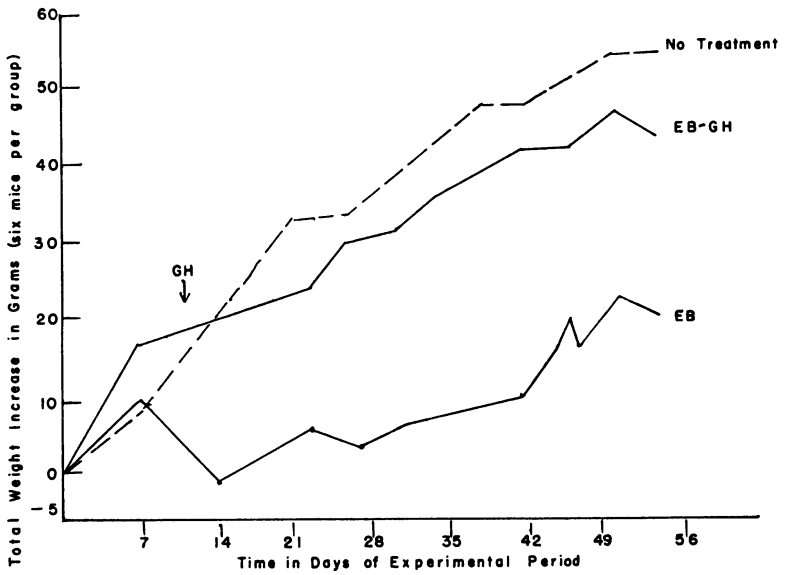


FIG. 2. Total increases in body weight of groups of six mice given large doses of estrogen (EB), large doses of EB + GH (growth hormone), or no treatment.

boundaries of the tips of the ducts or lobules. The total number of gland image cut-outs from each mouse were weighed, and the total weight was divided by the number of glands present to give the average "size" of the mammary glands of each mouse in "weight units." The reasons for using the average mammary gland response rather than the total response were 1) the numbers of mammary rudiments in different male mice range from two to six, 2) some mammary rudiments are refractory under all conditions, and 3) some glands may be damaged in preparation. This method of appraising mammary area did not give the emphasis that measurements of length times width would to glands of irregular shapes (for example, glands with one unusually long duct).

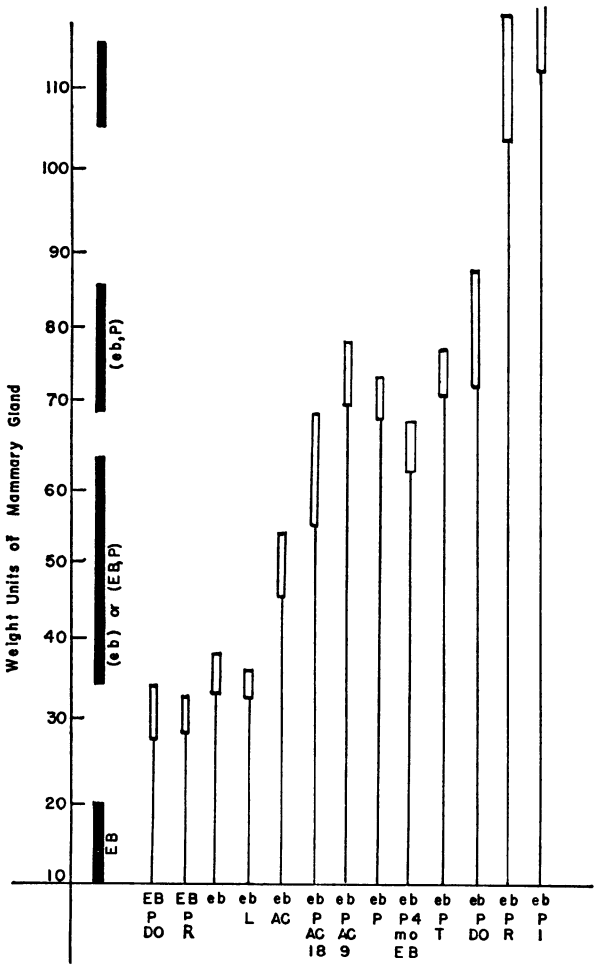


FIG. 3. Relative sizes of mammary glands of mice given different combinations of hormone treatment (EB, large doses of estradiol benzoate; eb, small doses of estradiol benzoate; L, lactogen; DO, desoxycorticosterone acetate; R, relaxin; ACTH, adrenocorticotropic hormone; T, testosterone; I, insulin). Open vertical bars represent standard error of the means of the treated groups.

OBSERVATIONS

Two major groups of experiments were undertaken. The first group was designed to determine whether the inhibitory effect upon the mammary gland of large doses of estrogens might be prevented by suitable supplementation with other hormones (Fig. 1). The second group of experiments was designed to determine whether the addition of other hormones to mice

given small or optimal doses of estrogens for mammary growth might inhibit mammary growth. Three types of control mammary glands were considered: 1) those of untreated male mice (Fig. 5); 2) those of mice given large amounts of estrogen (Fig. 6); and 3) those of mice given smaller amounts of estrogen (Fig. 7).

The mammary rudiments of the untreated male mice were uniformly small, an indication that they were not exposed to estrogen by contacts or through food. Mice given large doses of estradiol benzoate (EB) had mammary glands averaging less than 20 weight units (Table 1; Figs. 1 and 6); whereas mice given 2 μg. of eb every other day had mammary

TABLE 1. THE SIZE OF MAMMARY GLANDS OF MICE GIVEN, OVER AN EIGHT-WEEK PERIOD, LARGE (EB) AND SMALL (eb) AMOUNTS OF ESTRADIOL BENZOATE, WITH OR WITHOUT GROWTH HORMONE (GH)

Treatment	*Mammary response in weight units*
EB	< 20
EB and GH	< 20
eb	64 ± 5.5
eb and GH	53 ± 3.5

glands that were more than three times as large (Figs. 1 and 7). These observations affirm earlier observations.[1,5]

Not only were the mammary glands of the mice given eb larger in size, as determined by the area occupied by the glands, but they were structurally different, in that they were essentially branched tubular structures with few or no alveoli and certainly no lobules (compare Fig. 7 with Figs. 8 and 9). The much smaller glands of the mice given EB had many alveoli collected together to form lobules.

Young mice given large amounts of estrogen (EB) stop increasing in body weight after about seven days. After five or six weeks a second increase in weight may occur, but it is slight and is largely due to increased urine retention. The possibility that the inhibition of mammary growth by EB paralleled general inhibition of body growth was studied by giving growth hormone to mice given EB. In this way essentially normal body growth ensued (Fig. 2) but the mammary glands were as small as in mice given EB alone. The inhibition of mammary growth was not associated with a general deficiency of somatotrophic hormone.

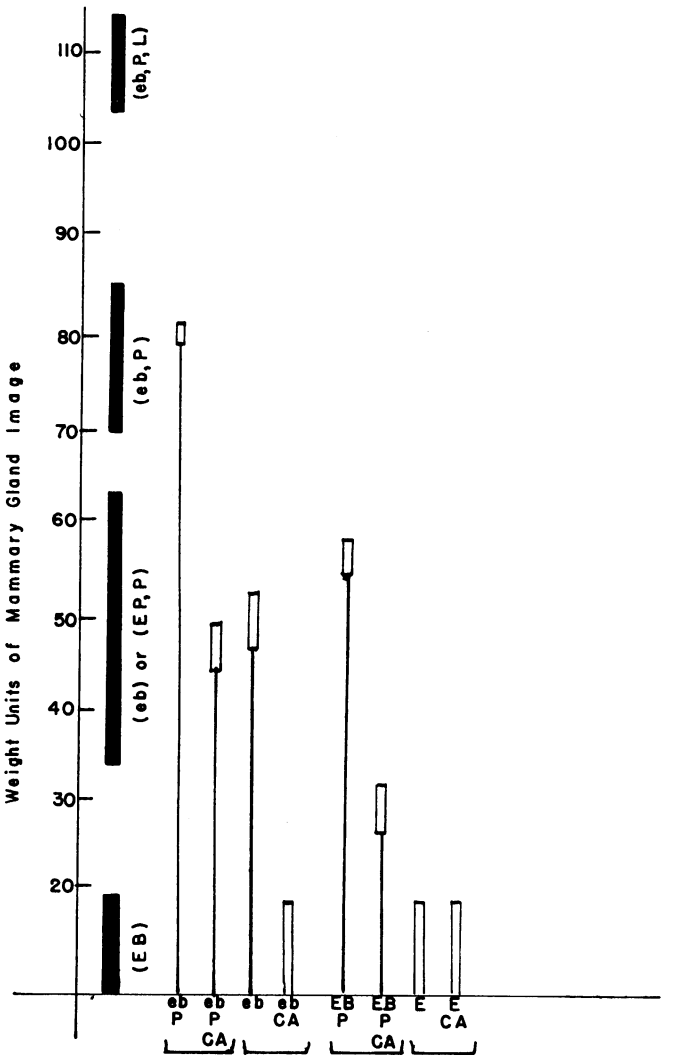
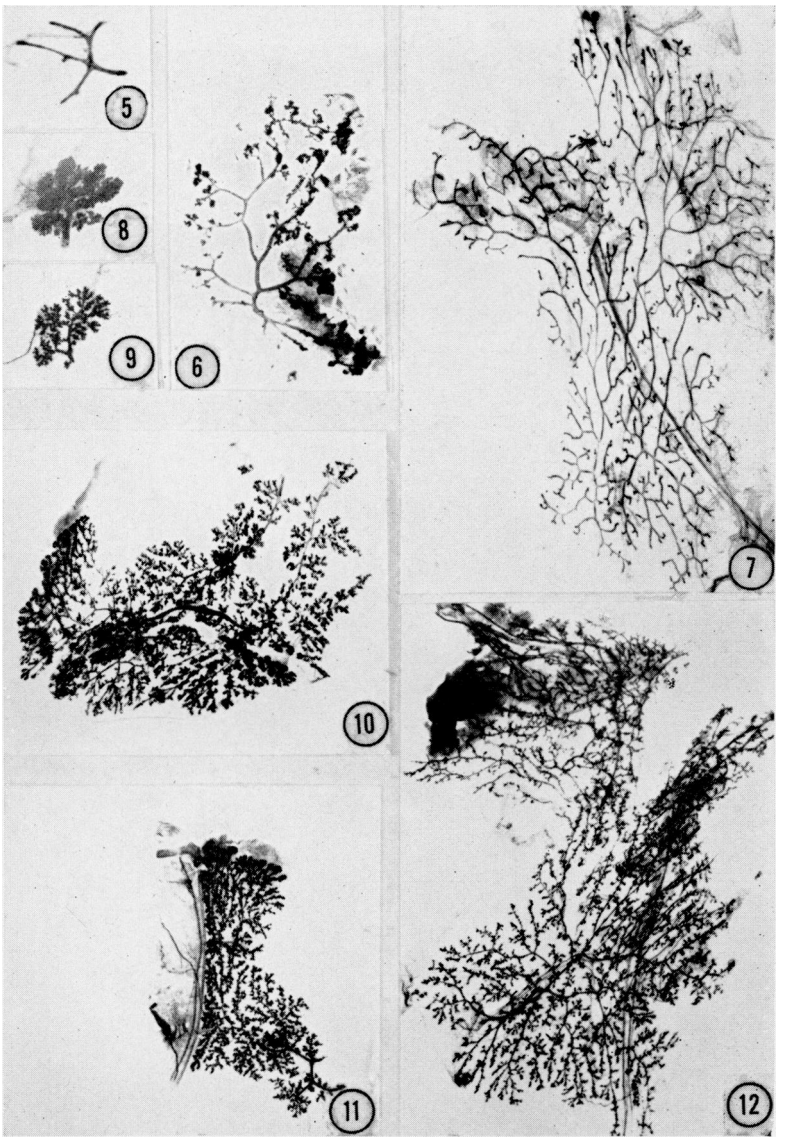


FIG. 4. Effects of cortisone acetate 125 μg./day on mammary size in mice also treated with estrogen, with or without progesterone.

The suprarenal glands of individual mice given EB ranged from 32 to 35 mg. per 100 gm. body weight, whether or not growth hormone was given, and were about 10 mg. per 100 gm. larger than those of mice given eb. The increased adrenal size was due to hypertrophy of the cortex, particularly the zona fasciculata. The bladders of the EB and the EB+GH treated mice were similarly distended secondary to obstruction at the



Photographs of Figs. 5-12 were taken at the same magnification (5x).

FIG. 5. Mammary gland of normal male mouse consisted of rudimentary ducts, devoid of smaller branching ducts or alveoli.

FIG. 6. Gland of male mouse was treated with high doses of estradiol benzoate (EB) for eight weeks. Many alveoli arranged in lobules were comparable to the lobules seen in the mammary gland of a mouse at parturition. Lack of small ductule formation, upon which alveoli are formed, led to a "spotty" distribution of alveoli, in contrast to the normal female gland. This gland was stunted in glandular area, as compared to Figure 7.

FIG. 7. Gland of male mouse, treated with eb for eight weeks. Alveolar growth was lacking in contrast to those of mice treated with EB. This gland was much larger with more numerous small ductule growth and numerous end budding.

FIG. 8. Mammary gland of male mouse treated with EB and L, 2.5 mg./day for 80 days. The mammary areas were scarcely larger than those of untreated male mice (Fig. 5). Alveolar growth was extensive, involving the complete glandular area, with secretion into the main duct.

FIG. 9. Gland of mouse given EB and P, and CA (cortisone). The gland resembled that shown in Figure 8.

FIG. 10. Gland of mouse treated with eb and P for 21 days after an initial treatment of EB for four months. There was considerable ductule and alveolar growth, similar to that of mice which were similarly treated but had not received the initial four-month EB.

FIG. 11. In addition to eb and P, this mouse also received CA. The gland was smaller but the number of small ducts and alveolar formations increased. Compare to Figure 12.

FIG. 12. Gland from mouse which received eb and P for three weeks. There was considerable small ductule growth, and early alveolar formation. Compare to Figure 11.

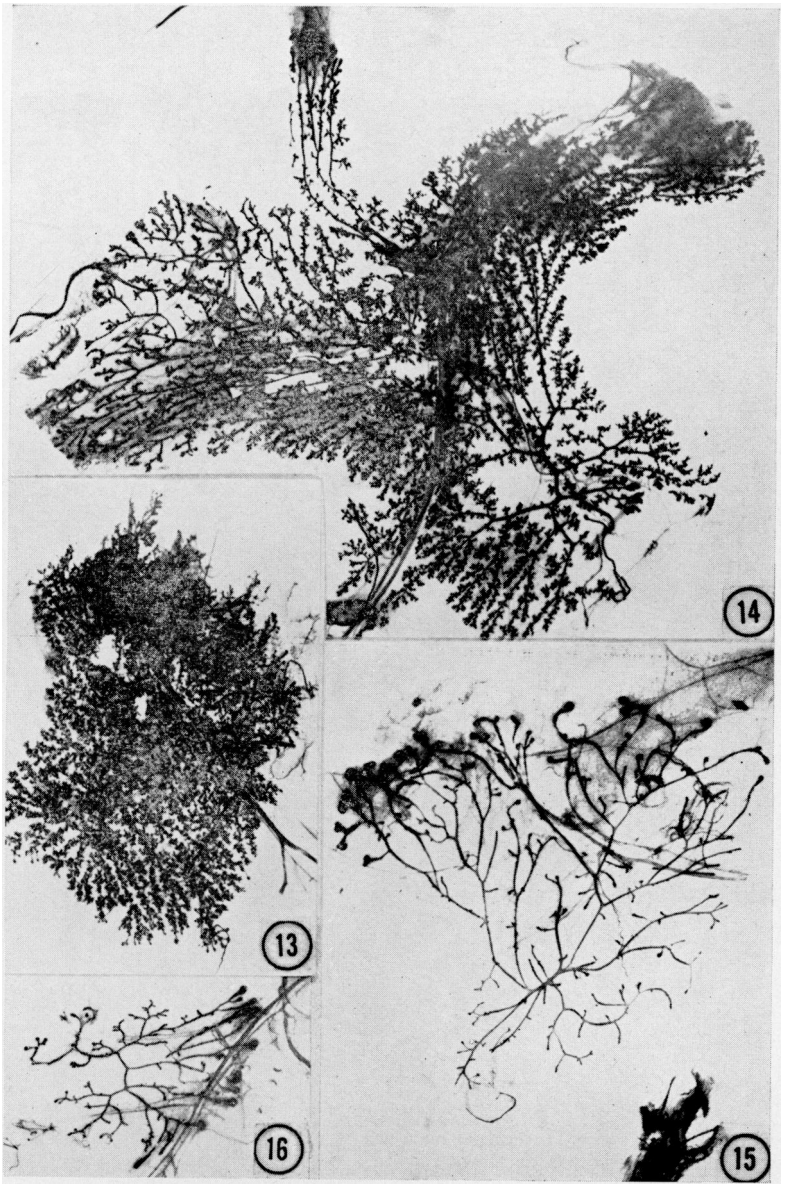


FIG. 13. Mammary gland from mouse given eb, P and ACTH (18 units in 24 days). Many lobules of alveolar were formed, beyond that seen in glands of mice not given ACTH. These glands resembled qualitatively glands of mice that also received CA (Figure 11).

FIG. 14. Mammary gland from mouse given eb, P, and 100 g.p. units of relaxin every four days, for three weeks. Similar glands developed in mice that received 0.5 units insulin/day or lactogen 1 mg./day, plus eb and P. These were the largest glands seen in this series, and showed small ductule and alveolar growth of an extensive degree. Compare to Figure 11.

FIG. 15. Gland from a mouse that received eb only. Growth was continuing as evidenced by the prominent end clubs and the beginning small ductule formation. Compare with the gland shown in Figure 7, from a mouse given eb and P for 21 days.

FIG. 16. Mammary gland from a mouse that received CA in addition to eb. Qualitatively the gland was similar to that shown in Figure 15 with large end clubs and some small ductule formation. However, the glandular area was smaller.

proximal part of the cavernous urethra. The testes and seminal vesicles of mice of both groups were small, indicating a decrease of gonadotrophic hormone; the pituitary glands were enlarged slightly.

The simultaneous injection of progesterone (P) with either EB or eb in young male mice resulted in larger mammary glands than in mice given EB or eb alone (Figs. 1, 12 and 15). The relative differences in size between the groups treated with EB and with eb were retained (Fig. 1). The mammary glands of EB+P-treated mice ranged from 31 to 56 weight units and those of the eb+P-treated mice 71 to 87 weight units. The mice given P as well as eb or EB had many more terminal ducts and alveoli, and some partial formation of lobules (Fig. 12).

Mammary glands of nine mice given eb and nine mice given eb+L were similar quantitatively and qualitatively (Fig. 3). Lactogenic hormone, when injected in amounts of 1 mg. daily, did not modify the mammary response to eb in intact young male mice. Furthermore, lactogenic hormone injected in amounts of 2.5 mg. daily for 80 days did not modify the mammary response of four male mice given EB (Fig. 8). The glands measured little larger than the untreated controls but consisted of small lobule-alveolar structures. Lactogenic hormone did not modify the growth inhibition attending EB treatment.

Six male mice were given pellets of methyltestosterone subcutaneously, and 45 days later were given eb and P for 21 days. The mammary response was the same as in mice given eb and P alone (Fig. 3).

Relaxin (R)+P+EB and R+P+eb were each given to groups of nine mice. The mammary response of the R+P+EB group were at the lower range of the weight unit scale of the EB+P group and the two groups were not significantly different from one another. The mammary glands of the mice given R+P+eb averaged about 110 weight units or over 30 weight units more than the mice given P+eb for comparable periods.

The mammary glands of a group of five mice given 250 μg. of desoxycorticosterone acetate daily with EB+P or eb+P did not differ significantly from the mice given EB+P or eb+P alone (Fig. 3). Similar groups of adrenalectomized mice given DO+EB+P or DO+eb+P had mammary glands comparable to those of intact mice similarly treated.

Twelve mice were given a total of nine units of ACTH over a 24-day period in addition to eb+P and 12 mice were given 18 units of ACTH +eb +P during a comparable period. The sizes of the mammary glands were not significantly different from those of the eb+P and EB+P controls (Fig. 3). The glands, however, were much better developed in that many lobules had developed and the amount of small terminal

branches had increased greatly (Fig. 13). Intrinsic adrenal cortical hormones seemed to augment the formation of alveoli.

P+eb+insulin (I) were given to 10 mice. The mammary glands of these mice were larger than those of mice given eb+P alone (Figs. 3 and 14). They resembled the glands of mice given relaxin. Not only were the glands large but the numbers of small terminal ducts and partially developed lobules were increased. Only one of 20 mice given P+EB+I survived a significant part of the experimental period. Although this one mouse had larger mammary glands than did mice given EB+P the number of observations is too limited to be conclusive.

An attempt was made to determine whether the mammary growth inhibitory effects of large amounts of estrogen (EB) would persist subsequent to discontinuation of treatment. Twelve mice were given EB for four months. After four months the pellets were removed and eb+P were given for three weeks. The glands were comparable to those of mice given eb+P alone for three weeks (Figs. 3 and 10). The mammary inhibiting effect of EB was not irreversible because normal mammary responses followed prolonged inhibition when more optimal hormonal environments ensued.

The mammary glands of mice given eb or eb and P plus cortisone acetate (Ca) were 40 to 70 per cent smaller than the glands of mice not given the corticoid (see Fig. 4; compare Figs. 11 and 12; Figs. 15 and 16). The mammary glands of the mice were given eb+P+Ca also had an increased number of terminal ducts and lobules of small alveoli; in this respect resembling the glands of mice that had been given eb+P for a longer period. The mice given eb+Ca, however, showed little or no alveolar development (Fig. 16). Cortisone seemed to augment the progesterone effect on stimulation of alveolar proliferation but not the duct proliferating effect of estrogens.

Mice given EB+P+Ca for three-week periods had small mammary glands but showed extensive alveolar proliferation (Fig. 9). The glands resembled those of mice given large amounts of estrogen plus lactogen (Fig. 8). Cortisone thus reduced the extent of duct proliferation occurring in mice given EB+P, tending especially to augment the effects of P (30 in contrast to 58 weight units, Fig. 4) and augment the alveolar proliferation.

Because the fatty mammary stroma around the terminal end buds of the proliferating ducts is more basophilic it was at least interesting to inject hyaluronidase (R) to determine its effects on mammary growth. Eleven mice were given either 1.5, 3.0 or 10 units per day of hyaluronidase

in addition to eb plus P for three weeks. The mammary glands were similar in size and type of development to those of mice not given hyaluronidase.

DISCUSSION

None of the hormones used prevented the restricted mammary growth of mice given large amounts of estradiol benzoate (EB). Mice given progesterone had larger mammary glands than did those given EB alone but not as large as those given smaller amounts of estrogen (eb) and progesterone (P). Mice given P had glands with more small ducts and alveoli. The only possible exception was that insulin-treated mice given EB+P might have had larger glands but the critical experiments could not be done. The pituitary growth hormone and lactogenic hormone did not reverse the mammary growth restriction of large amounts of estrogen, but mice given these hormones had small glands with alveoli.

The mammary glands of young male mice given eb+P were larger than those given eb alone and the effects of the added Ca largely inhibited the effects of P on ductal proliferation. However, the glands of mice given eb+Ca resembled those of mice given EB. Cortisone acetate in amounts of 125 μg. daily was the only hormone given that when given in addition to small amounts of eb resulted in mammary growth approximately that seen in mice given EB. It seems improbable that the mechanism whereby large amounts of estrogen inhibit mammary growth is through the adrenal glands. Adrenalectomized mice given eb or EB plus desoxycorticosterone had mammary glands comparable to the controls given eb or EB. The large doses of cortisone acetate may have acted as did the large doses of estrogen. Cortisone seemed less detrimental to the proliferation of smaller ducts and alveoli than it did to the growth of the larger ducts and in this manner again resembled EB. Both substances were given at toxic levels and hence the responses should probably be considered in a pharmacological connotation rather than a physiological one.

Although estrogens provoke little or no mammary growth in hypophysectomized mice they did induce growth of subjacent mammary tissue when applied in very small amounts to the skin, and remote glands were not influenced. [8, 9, 10] Small amounts of estrogens incite local mammary growth in the presence of circulating levels of pituitary hormones and hence seem to have a direct action on mammary tissue, but a dependent one.

It is probable that the large doses of estrogen may directly inhibit mammary growth. Mammary growth is not inhibited in the same way as somatic growth because the inhibitory effects of large amounts of estrogen on somatic growth were prevented by growth hormone, but the mammary glands remained small.

The mammary glands consist of both parenchyma and stroma. The stroma is usually considered to be passive in most epithelial proliferative processes. The mice experiments of Nandi[11] have revealed that fatty stroma is essential for the proliferation of hyperplastic mammary nodules and the same is also true for transplanted segments of mammary ducts.[13] The site of action of estrogens in large amounts and of cortisone in large amounts in the restriction of mammary proliferation may be upon the mammary stroma rather than directly on the mammary parenchyma. The influences of insulin and relaxin in augmenting the mammary responses might be interpreted as acting on the stroma.

SUMMARY

1. Male mice given large doses of estradiol benzoate show little extension of the mammary area but some increase in small ducts and a few alveoli.

2. The injection of growth hormone, lactogen, relaxin, hyaluronidase, adrenocorticotrophic hormone or desoxycorticosterone in addition to large doses of estradiol benzoate did not modify the mammary response. Mice given progesterone in addition to large doses of estradiol benzoate had slightly larger mammary glands than those given EB alone.

3. Mammary growth of mice given smaller and near optimal amounts of estrogen was inhibited by daily injections of 0.125 mg. of cortisone acetate and resembled the glands of mice given large amounts of EB.

4. That large doses of estrogen did not inhibit mammary growth by augmenting adrenal cortical function was indicated by 1) the similarity of large and small doses of estradiol benzoate on the mammary glands in adrenalectomized and intact mice given desoxycorticosterone, and 2) effects of adrenocorticotrophic hormones when given in combination with either large or small doses of estrogens.

5. Mice given either insulin, relaxin or lactogen in addition to small doses of estrogen plus progesterone had larger glands than those given estrogen and progesterone alone.

6. The over-all area of the mammary responses was determined quantitatively by a "paper weight" method.

REFERENCES

1. Gardner, W. U., Smith, G. M., and Strong, L. C.: Stimulation of abnormal mammary growth by large amounts of estrogenic hormone. *Proc. Soc. exp. Biol. (N.Y.)*, 1935, *33,* 148-150.
2. Gardner, W. U. and Chamberlain, T. L.: Local action of estrone on mammary glands of mice. *Yale J. Biol. Med.*, 1941, *13,* 461-465.
3. Geschickter, C. F. and Hartman, C. G.: Mammary gland response to prolonged estrogen stimulation in the monkey. *Cancer (N. Y.)*, 1959, *12,* 767-781.
4. Sharf, G. and Lyons, W. R.: Effects of estrone and progesterone on male rabbit mammary glands. II. Varying doses of estrone. *Proc. Soc. exp. Biol. (N.Y.)*, 1941, *48,* 86-89.
5. Gardner, W. U.: Inhibition of mammary gland growth by large amounts of estrogen. *Endocrinology,* 1941, *28,* 53-61.
6. Astwood, C. F., Rausch, E. O., and Geschickter, C. F.: Development of the mammary gland of the rat. *Amer. J. Anat.*, 1937, *61,* 373-405.
7. Van Heuverswyn, J., Folley, S. J., and Gardner, W. U.: Mammary growth in male mice receiving androgens, estrogens, and DOCA. *Proc. Soc. exp. Biol., (N.Y.)*, 1939, *41,* 389-392.
8. Huseby, R. A.: Estrogen therapy in the management of advanced breast cancer. *Amer. J. Surg.*, 1954, *20,* 112-124.
9. MacBryde, C. M.: The production of breast growth in the human female. *J. Amer. med. Assoc.*, 1937, *112,* 1045-1049.
10. Lyons, W. R. and Sako, Y.: Direct action of estrone on the mammary gland. *Proc. Soc. exp. Biol. (N.Y.)*, 1940, *44,* 398-401.
11. Nandi, S.: Hormonal control of mammogenesis and lactogenesis in the C_3H/He Crgl mouse. *Univ. Calif. Publ. Zoöl.*, 1959, *65,* 1-128.
12. Hoshino, K. and Gardner, W. U.: Growth potentialities of the normal mammary tissue in mice. *Anat. Rec.*, 1961, *139,* 240 (Abst.).